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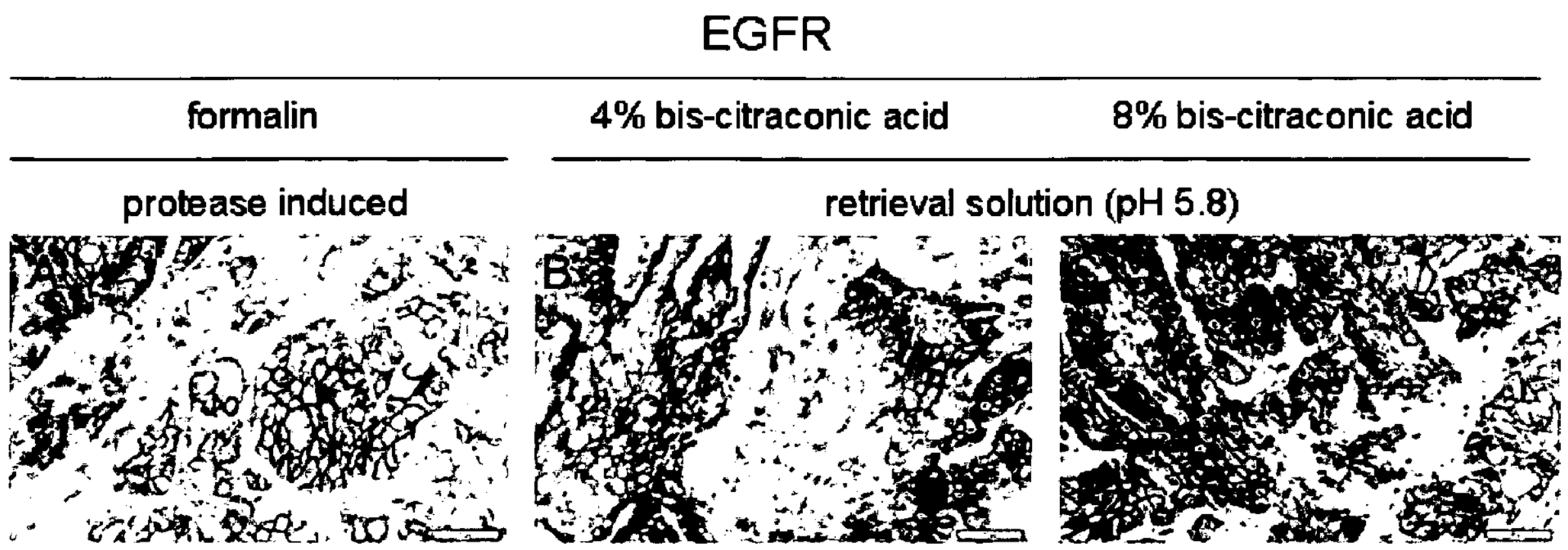
(54) **Titre : UTILISATION D'UN AGENT DE RETICULATION DE TYPE ANHYDRIDE BIS-MALEIQUE POUR LA FIXATION D'UN ECHANTILLON DE CELLULE OU DE TISSU**

(54) **Title: USE OF A BIS-MALEIC ANHYDRIDE CROSS-LINKING AGENT FOR FIXATION OF A CELL OR TISSUE SAMPLE**



(57) **Abrégé/Abstract:**
The present invention relates to novel bis-maleic anhydrides. It especially relates to the discovery that bis-maleic anhydride cross-linking agents can be used for preservation/fixation of a cell or tissue sample. With great advantage a bis-maleic anhydride cross-linking agent can be used in methods requiring fixation of a cell or tissue sample and at the same time requiring that the fixative has little impact on the later detection of a protein or a nucleic acid in procedures like immunohistochemistry, fluorescence in situ hybridization or RT-PCR.

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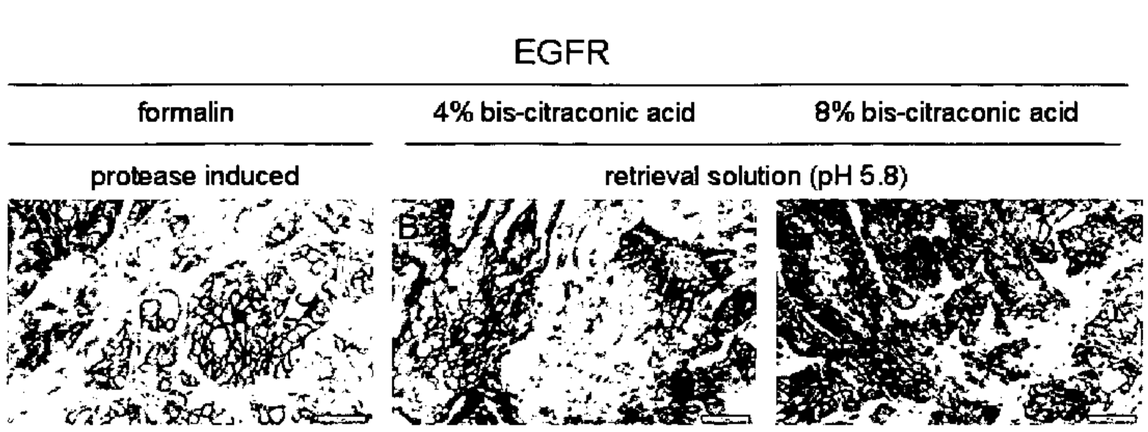
(54) Title: USE OF A BIS-MALEIC ANHYDRIDE CROSS-LINKING AGENT FOR FIXATION OF A CELL OR TISSUE SAMPLE

Fig. 2



(57) Abstract: The present invention relates to novel bis-maleic anhydrides. It especially relates to the discovery that bis-maleic anhydride cross-linking agents can be used for preservation/fixation of a cell or tissue sample. With great advantage a bis-maleic anhydride cross-linking agent can be used in methods requiring fixation of a cell or tissue sample and at the same time requiring that the fixative has little impact on the later detection of a protein or a nucleic acid in procedures like immunohistochemistry, fluorescence in situ hybridization or RT-PCR.

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Use of a bis-maleic anhydride cross-linking agent for fixation of a cell or tissue sample

Background of the Invention

The present invention relates to novel bis-maleic anhydrides. It especially relates to the discovery that bis-maleic anhydride cross-linking agents can be used for preservation/fixation of a cell or tissue sample. With great advantage a bis-maleic anhydride cross-linking agent can be used in methods requiring fixation of a cell or tissue sample and at the same time requiring that the fixative has little impact on the later detection of a protein or a nucleic acid in procedures like immunohistochemistry, fluorescence in situ hybridization or RT-PCR. It is also demonstrated that the use of a bis-maleic anhydride cross-linking agent as a fixative greatly facilitates later detection of an analyte of interest in a previously fixed cell or tissue sample.

To date there is no generally applicable, "ideal" way to prepare a cell or tissue sample, e.g. for immunohistochemistry or detection of a nucleic acid of interest, respectively. Fixation and the reversibility of negative effects introduced by the fixation have a major impact on the detectability of polypeptide antigens and nucleic acids, respectively, and on the reproducibility of the results obtained thereupon.

For successful immunostaining of an antigen in a cell or tissue sample at least three criteria have to be met: a) retention of the antigen at its original site, b) accessibility of the antigen and c) correct conformation/preservation of the antigen/epitope of interest. It would appear that at present no fixation and/or detection procedure fully meets all these three criteria. For the procedures known in the art, best performance for one or two of these criteria goes to the expense of a reduced performance in at least one other criterion.

Several fixatives are available and used in the routine of a clinical pathology laboratory, like glutardialdehyde, formaldehyde and acetone, or other organic solvents. The vast majority of fixation procedures, however, are based on the use of cross-linking agents, like formaldehyde. The fixative solution usually is an aqueous formaldehyde solution that contains sodium phosphates, contrived to provide buffering (minimal pH change following addition of a small amount of strong acid or base) to pH 7.2-7.6 and an approximately isotonic solution (one whose osmotic

pressure is the same as that of mammalian extracellular fluids, often based on physiological saline).

With state-of-the-art procedures fixation has to be just right.

If fixation is too short, instead of fixation merely coagulation of proteins by the alcohols used to dehydrate the sample occurs. This may e.g. negatively impact the preservation of tissue morphology or impair long term storage stability.

With prolonged formaldehyde fixation the cross-linked protein molecules form a dense network that can impair the penetration of paraffin wax or/and the access of antibody molecules. As a result an antigen of interest may be reversibly or even irreversibly masked. Further an epitope may be chemically modified ("destroyed") e.g., by reaction with formaldehyde.

In addition, it is known that the activity of most enzymes is impaired after formaldehyde fixation.

As mentioned before, fixation in formaldehyde is most widely used in clinical pathology. The major reason most likely is that by fixation with formaldehyde the antigen of interest is trapped at the sites it occupied in the living organism. By way of methylene bridges introduced upon formaldehyde fixation also the morphology of a cell or tissue sample is well preserved. These positive effects, however, go to the expense of permeability of the sample and to the fixation causing changes in the accessibility and/or conformation of an antigen/epitope of interest, damage in nucleic acids and inactivation of enzyme activity.

Cross-linking due to formaldehyde fixation is likely to mask or to destroy epitopes, leading to a false negative immunostaining. This failure is even more likely to occur when the primary immunoreagent is a monoclonal antibody than when a polyclonal antiserum is used. This is why many, many attempts have been made and are found in the relevant literature dealing with reversing the effects of formaldehyde fixation.

For long term storage a fixed cell or tissue sample usually has to be de-hydrated and embedded in an appropriate embedding medium. Paraffin embedding is usually preferable to either plastic embedding or cutting un-embedded specimens with a vibrating microtome or in a cryostat.

As illustrated above, all fixation procedures to a certain extent represent compromises of various kinds. Often optimal preservation of morphology goes to the expense of accessibility for an antibody or destruction of an antigen or of an epitope thereon.

However and also important to the present invention, not only is there a high variability introduced during preparation of a specimen, like its fixation or further processing like embedding with paraffin, probably even more variability is caused by the various modes and routes of regaining immunological reactivity or accessibility in detection of nucleic acids, i.e. in procedures known as antigen retrieval.

Despite the broad use and great utility of e.g. immunohistochemical methods or methods for detecting a nucleic acid of interest in a cell or tissue sample there is great need for further improvements. Such improvements may for example relate to more gentle fixation of a cell or tissue sample, to improvements in antigen retrieval or/and to better comparability and reproducibility of results and may be even to the possibility to use antibodies for which the corresponding antigen or epitope is destroyed in standard procedures, like formaldehyde fixation.

The inventors of the present invention have surprisingly found that the use of bis-maleic anhydrides as a cross-linking agent in the preparation/fixation of a cell or tissue sample is of tremendous advantage and can and will lead to significant improvements regarding at least one or even several of the problems known in the art.

Summary of the Invention

The present invention relates to a method for fixation of a cell or a tissue sample in vitro wherein said cell or said tissue sample is incubated with a bis-maleic anhydride cross-linking agent, whereby said cell or said tissue sample is fixed.

Further disclosed is a method of preserving a cell or a tissue sample the method comprising the steps of fixing a tissue sample with a bis-maleic anhydride cross-linking agent and of embedding said fixed sample in paraffin.

The present invention also discloses a method for performing immunohistochemistry on a cell or a tissue sample the method comprising the steps of fixing a cell or tissue sample with a bis-maleic anhydride cross-linking agent,

embedding said fixed sample in paraffin, de-paraffinizing said sample, removing the bis-maleic amide cross-link and immunologically detecting an epitope of interest.

Also described is a method for detecting in vitro a nucleic acid of interest by in situ hybridization on a cell or a tissue sample the method comprising the steps of fixing a cell or tissue sample with a bis-maleic anhydride cross-linking agent, embedding said fixed sample in paraffin, de-paraffinizing said sample, removing the bis-maleic amide cross-link and detecting a nucleic acid of interest by in situ hybridization.

Further a method for detecting in vitro a nucleic acid of interest by RT-PCR in a cell or a tissue sample the method comprising the steps of fixing a cell or tissue sample with a bis-maleic anhydride cross-linking agent, embedding said fixed sample in paraffin, de-paraffinizing said sample, removing the bis-maleic amide cross-link and detecting a nucleic acid of interest by performing RT-PCR is given.

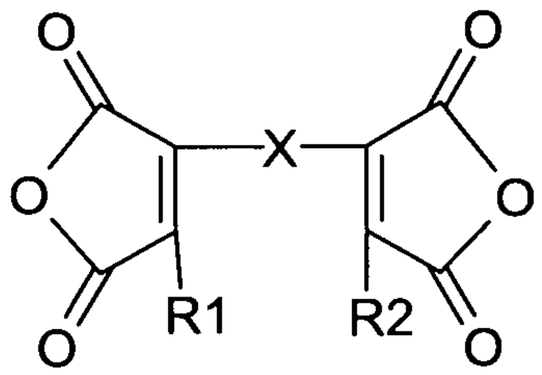
It is also shown that based on a method according to the present invention both a polypeptide of interest and a nucleic acid of interest can be detected in the same specimen prepared from a cell or tissue sample. The invention relates to a method for detecting in vitro at least one polypeptide of interest by immunohistochemistry and at least one nucleic acid of interest in one test sample comprising a cell or a tissue sample, the method comprising the steps of fixing a cell or tissue sample with a bis-maleic anhydride cross-linking agent, embedding said fixed sample in paraffin, de-paraffinizing said sample, removing the bis-maleic amide cross-link and immunologically detecting the at least one polypeptide of interest and detecting the at least one nucleic acid of interest by performing RT-PCR or fluorescence in situ hybridization.

The invention further relates to the use of a bis-maleic anhydride cross-linking agent for fixation of a cell or a tissue sample as well as to the use of a bis-maleic anhydride cross-linking agent in the manufacturing of a fixative for fixation of a cell or tissue sample.

Detailed Description of the Invention

In a first embodiment the present invention relates to a method for fixation of a cell or a tissue sample in vitro wherein said cell or said tissue sample is incubated with a bis-maleic anhydride according to Formula I,

Formula I



wherein R1 and R2 independently are selected from the group consisting of hydrogen, methyl, ethyl, propyl, isopropyl and butyl, wherein X is a linker with between 1 and 30 atoms in length and whereby said cell or said tissue sample is fixed.

The compound according to Formula I sometimes will simply be referred to as "bis-maleic anhydride cross-linking agent" or "bis-maleic anhydride".

It will be understood that "a method for fixation of" in the sense of the present invention is equivalent to "a method of treating", that "fixing" is equivalent to "cross-linking", "is fixed" could alternatively phrased as "is cross-linked" and that "fixed" by and in a method of the present invention relates to "comprising a bis-maleic amide cross-link" or "comprise(s) a bis-maleic amide cross-link". For the sake of convenience and in light of the fact that the skilled artisan is fully aware of the meanings attached to terms like fixation, fixative or fixed, and for the sake of convenience, only these terms will generally be used throughout the description.

It will also be appreciated that in a scientifically correct sense it is not a cell or a tissue sample that is fixed or cross-linked but rather it is the biomolecules contained in such sample that are cross-linked or fixed in a fixation method as disclosed in the present invention. The cross-links in these biomolecules may be intra-molecular as well as intermolecular cross-links.

If at least two maleic anhydrides, linked to each other by a linker X (at least a bis-maleic anhydride), are reacted with at least two primary amines at least two amide bounds are formed and the at least two primary amines are cross-linked via the at least two amide bonds and via the linker X. For the sake of convenience this type of cross-link in the following will be referred to as "bis-maleic amide cross-link".

The articles "a" and "an" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article.

The expression "one or more" or "at least one" denotes 1 to 20, preferably 1 to 15 also preferred 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 12.

The present invention is based on the surprising and striking discovery that a bis-maleic anhydride can be used to gently and reversible fix a cell or a tissue sample. Without being wanted to be bound to this theory the great advantages are believed to be due to the facts that a) a bis-maleic anhydride cross-linking agent rapidly and effectively forms bis maleic amide cross-links, that b) the cross-linking agent bis-maleic acid can be easily removed whenever desired and/or as may also be that c) by using a linker X of an appropriate length less negative effects like distortion or destruction of epitopes are likely as compared to the relatively short cross-linker formaldehyde.

To fully appreciate the tremendous advantages of the method according to the present application it will be helpful to discuss in more detail those tissue fixation procedures that are most frequently used, i.e. procedures based on the use of formaldehyde as a fixative.

A formaldehyde-based fixative is usually derived from formalin, which is a solution containing 37% w/w (= 40% w/v) formaldehyde in water. The working fixative is a ten-fold dilution of formalin (4 grams per 100 ml). A solution of almost identical composition may be made with paraformaldehyde as the starting material. Paraformaldehyde is a solid polymer that changes into formaldehyde when heated (in slightly alkaline water) to 60 °C. Though the phrase "fixed in 4% paraformaldehyde" is often used in the literature it is not fully correct. Most of the formaldehyde in a diluted aqueous solution is present as methylene glycol, which is formed by addition of a molecule of water to one formaldehyde molecule:

$$HCOH + H2O \leftrightarrow H2C(OH)2$$

The concentration of free formaldehyde in the fixative solution is very low. Nevertheless, it is free formaldehyde, rather than methylene glycol, that enters the chemical reactions of fixation.

The chemical reactions of fixation by formaldehyde are primarily with primary amines, as e.g. present in polypeptides.

Formaldehyde fixation does appear to be a two-step process. In a first step formaldehyde is rapidly bound. By this rapid binding formaldehyde probably stops

autolysis but it does little to stabilize the fine structure of the tissue, and does not provide effective protection against disruptive effects of later treatments such as paraffin embedding.

In the first stage (hours), formaldehyde molecules combine with various parts of protein molecules, especially the side-chain amino group of lysine and the nitrogen atoms of peptide linkages:

Protein –NH2 + H2C(OH)2 ↔ protein – NHCH2OH + H2O.

In the second step the bound hydroxymethyl groups react with other nitrogen atoms of the same or adjacent protein molecules, thereby generating a methylene cross-link or bridge. These methylene (—CH2 —) bridges, are stable and account for the insolubility and rigidity of protein-containing tissues that have been fixed by formaldehyde. One possible reaction is:

Protein-NH-CH2OH + NH2-protein → protein-NH-CH2-NH-protein + H2O

In addition to the reactions with proteins, formaldehyde may also combine with some basic lipids.

It is likely that the above discussed two steps of chemical reactions required for "full" formaldehyde fixation account for some of the difficulties encountered when working with formaldehyde-fixed material. Brief exposure to formaldehyde does not cause sufficient cross-linking to immobilize small proteins or other small analytes. A too long fixation can cause irreversible damages especially due to formation of an excess of methylene bridges. It is generally accepted that for reasonable structural preservation a specimen should remain in a formaldehyde solution at least over night or even for about 24 hours.

In immunohistochemistry the epitopes of an antigen of interest must be accessible to the primary antibody. An epitope is a small part of a large molecule, such as an amino acid sequence of about 5 to about 10 amino acids that specifically binds to the binding site of an antibody molecule. A monoclonal antibody recognizes only one epitope. A polyclonal antiserum, on the other hand may recognize several different epitopes.

Reversing the unwanted negative side effects of e.g. formaldehyde fixation is known in the literature under headings like antigen retrieval or epitope retrieval.

At least three different routes for antigen retrieval are broadly used alone or on combination: partial enzymatic digestion, heat and/or different chemicals supposed to reverse formaldehyde effects.

For partial proteolytic digestion e.g. an inexpensive grade of porcine trypsin (containing some chymotrypsin) is used. The rationale of using a proteolytic enzyme is that breaking some peptide bonds will make holes in the matrix of cross-linked proteins, allowing the entry of e.g. antibody molecules. However, proteolytic enzymes invariably attack all proteins, including the antigen of interest. The tight-rope walk is to digest just long enough and as one can easily appreciate the conditions for digestion will vary from tissue to tissue and/or from antigen to antigen.

Most of the formaldehyde bound to a fixed tissue can be removed by heat induced antigen retrieval. However, heat can also cause the irreversible destruction of target analytes. Heat is e.g. known to destroy heat-labile epitopes or heat-labile enzymes.

Heat induced antigen retrieval is often combined with use of special "retrieval" or "extraction" buffers. However, success of these procedures can no way be predicted and the unpredictable outcome of such procedure is one of the great mysteries in the field of e.g. immunohistochemistry.

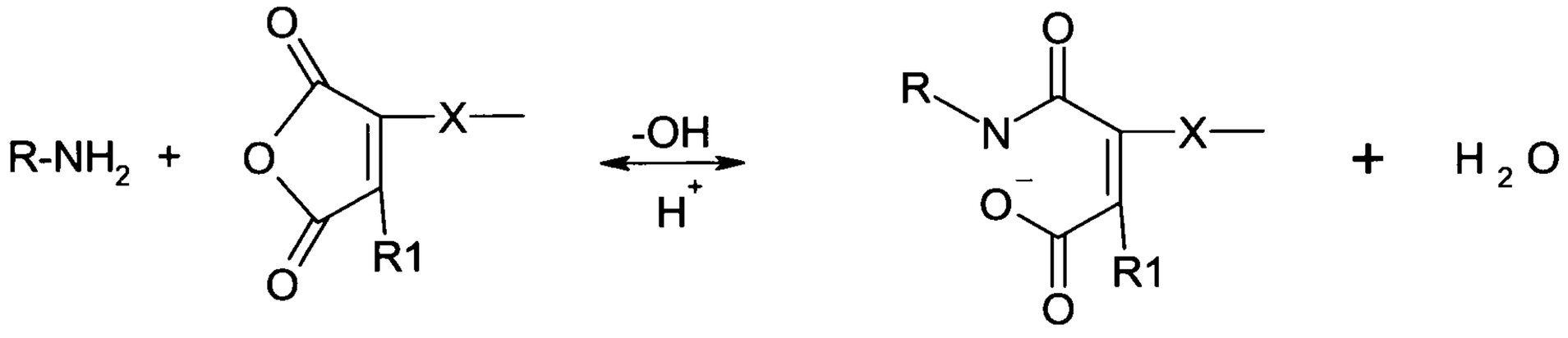
Many different buffers and pH-values (e.g. citrate; glycine/HCl – mainly for acidic pH-values ranging from about 2.5 to about 6 and alkaline buffer on basis of Tris in the pH-range of about 9 to 10) have been used, either alone or in combination with chemicals believed to reverse at least partially the negative effects of the methylene cross-links introduced by formaldehyde. Chemicals used either alone dissolved in water, or dissolved in buffer e.g. are EDTA, citraconic acid, lead thiocyante, aluminum chloride, or zinc sulfate.

The large variety of ingredients used in solutions for high temperature antigen retrieval indicates that more than one mechanism is probably involved. Most antigens can be retrieved at near neutral pH, but a more alkaline medium is needed for some and acidic conditions for others. In certain cases bonds to tissue-bound calcium ions may mask epitopes, necessitating removal of the metal ions by chelation. Other ingredients of retrieval solutions include heavy metal ions, which may expose epitopes by a coagulation-like action on proteins, and chaotropic

substances which may modify the shapes of proteins by changing the structures of clusters of water molecules.

For analysis of nucleic acids, e.g. from formaldehyde-fixed paraffin-embedded (FFPE) tissue various methods of antigen (analyte) retrieval, often quite different to the ones required for immunological detection of an epitope of interest, are recommended and used in the art. Nonetheless it is also accepted that formaldehyde fixation may have a negative impact on nucleic acids. For example, it is known that messenger RNA (m-RNA) in FFPE tissue is at least partially destroyed, rendering the detection of m-RNAs of more the 100 nucleotides in length a quite challenging task.

Coming now to the properties and advantages of a bis-maleic anhydride cross-linking agent as shown in the present application: The reaction of maleic anhydride with a primary amine can be depicted by the following reaction scheme:



It is important that on the one hand the amide bond formed is rather stable during (long term) storage of a sample fixed on the basis of such reagent and that on the other hand the amide bonds can be easily broken and that the primary amines can be regained easily and under gentle conditions.

Amide bond formation between a primary amine group and a maleic anhydride group occurs rapidly at neutral and alkaline pH. Preferably the incubation of a cell or tissue sample with a bis-maleic anhydride according to Formula I is performed at a pH of 7.0 or above. Also preferred the pH is below pH 12. Further preferred the pH used for fixation is in the range of pH 7.0 to pH 11.0, including the boarders. Also preferred the incubation is performed at a pH of 7.5 to 10.0, the boarders inclusive. For obvious reasons a buffer substance comprising a primary amine should be avoided.

Preferably the fixative will not only be buffered to stabilize the pH during fixation, but the buffer will also have a physiological salt concentration. It is further advantageous and preferred, if the bis-maleic anhydride cross-linking agent is provided in a buffer also comprising a water-miscible organic solvent. Preferred organic solvents in this context are ethanol, N,N'-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO).

The amide bound formed by reaction of maleic anhydride with a primary amine is stable at alkaline pH. If stored, a sample fixed with a bis-maleic anhydride according to Formula I preferably is kept at neutral or an alkaline pH, with the same preferred ranges as given above for the fixation or incubation step.

A sample comprising cells in suspension or a small tissue sample of 1 μm^3 or less can be fixed within short time, like, e.g. within 10 to 60 min. In clinical routine a tissue sample, however, will often be much larger than 1 μm^3. For routine purposes it is therefore preferred that a cell or tissue sample is incubated for 1 to 72 hours with the bis-maleic anhydride cross-linking agent. As the artisan would say the cell or tissue sample is fixed for 1 to 72 hours. It is also preferred that in a method according to the present invention the incubation of the cell or tissue sample in the fixative comprising a bis-maleic anhydride according to Formula I is performed for 2 to 48 hours.

A bis-maleic anhydride cross-linking agent may e.g. be dissolved in a organic solvent at a high concentration and can the be diluted to yield an appropriate working or final concentration. The final concentration of the bis-maleic anhydride cross-linking agent used in the incubation/fixation step may vary to some extend. Preferably the final concentration is between 0.1 and 20 %. Further preferred the final concentration will be between 0,25 and 10 % (% in weight per volume).

The amide bond formed between a primary amine group and a maleic anhydride group in a fixation method as disclosed in the present application can rapidly be broken under acidic buffer conditions. Upon incubation in acidic pH-buffers the amide bond is broken, the bis-maleic amide cross-linking is removed and the bis-maleic anhydride can be washed away. At the same time the primary amine previously part of an amide bond with the cross-linking agent is regained and present again. Preferably a bis-maleic amide cross-link is removed by incubating a fixed sample, i.e. a sample fixed by use of a bis-maleic anhydride according to Formula I, under acidic pH. Preferably a bis-maleic amide cross-link is removed by

incubation in a buffer with a pH from 2.0 to 6.5. Also preferred the buffer used for removing a bis-maleic amide cross-link will have a pH from 2.5 to 6.5, or from 3.0 to 6.0, wherein each of the boarders is inclusive. Removal occurs rapidly, the more rapid, the more acidic the buffer. Preferably the fixed sample is incubated for 2 min to 2 hours, also preferred from 5 min to 1 hour in order to remove a bis-maleic amide cross-link.

It has been found that the residues R1 and R2 have to be selected from hydrogen, methyl, ethyl, propyl, isopropyl or butyl for use of the bis-maleic anhydride cross-linking agent in a method disclosed in the present application.

Further preferred, the residues R1 and or R2 according to Formula I are selected from hydrogen, methyl or ethyl. Also preferred the bis-maleic anhydride for use in a method according to the present invention is a bis-maleic anhydride according to Formula I, wherein R1 is hydrogen or methyl and R2 is hydrogen or methyl.

If R1 and R2 are the same the amide bonds formed during fixation of a cell or tissue sample can be reversed under the same conditions. This will be advantageous whenever as much as possible cross-linker shall be removed and at the same time as much as possible primary amine shall be regained. In a preferred embodiment the bis-maleic anhydride for use in a method according to the present invention therefore is a bis-maleic anhydride according to Formula I, wherein R1 and R2 are the same.

As the skilled artisan will appreciate, now that it has been found that a bis-maleic anhydride can be used for fixation of a cell or tissue sample many, many compounds comprising at least two maleic anhydrides linked by a linker can be designed and used.

The linker X in the bis-maleic anhydride for use in a method according to the present invention has a length of between 1 and 30 atoms. The term length must understood as consisting of the number of atoms given. A linker of 30 atoms in lengths has a backbone that consists of 30 atoms.

Obviously the linker X can be designed in many ways as the specific application may require and no undue limitation or restriction would be appropriate. Nonetheless some preferred examples of such linkers shall be given.

In one preferred embodiment the bis-maleic anhydride according to Formula I for use in a method according to the present invention will have a linker X with a backbone consisting of carbon atoms and optionally one or more heteroatom(s) selected from O, N and S. Also preferred the heteroatoms comprised in the backbone of the linker X will be either O or N or both.

The linker X may carry side chains. In a preferred embodiment the bis-maleic anhydride according to Formula I for use in a method according to the present invention will have a linker X having one or more side chains designed to carry one or more further maleic anhydride group or groups, respectively. Preferably the bis-maleic anhydride according to Formula I for use in a method according to the present invention will have a linker X with one to three maleic acid groups attached to one or more side chain, resulting in a compound according to Formula 1 with 3 to five maleic anhydride groups.

Preferably the linker X in a compound according to Formula I for use in a method according to the present invention will have a molecular weight of 10 kD or below. Also preferred, the linker X will have a molecular weight of 5 kD or below, of 3 kD or below, of 2 kD or below, or of 1 kD or below. In one preferred embodiment the bis-maleic anhydride cross-linking agent for use in a method according to the present application will have a molecular weight of 1 kD or below.

While it is possible to design and use maleic anhydride cross-linking agents comprising three, four, five or even more maleic anhydride groups, it is preferred to use a bis-maleic anyhydride cross-linking agent having exactly two maleic anhydride groups that are linked by the linker X.

Whereas in some applications side chains in the linker X will be of utility in other applications, a linker X having a backbone without side chains will be preferred. A linker without side chains is a linker having only the atoms of the backbone and atoms that are directly bound to the atoms of the backbone.

In one preferred embodiment the bis-maleic anhydride according to Formula I for use in a method according to the present invention will have a linker X of 1 to 20 atoms in length.

In one preferred embodiment the linker X in the bis-maleic anhydride according to Formula I for use in a method according to the present invention is selected from the group of linkers consisting of a linker of 1 to 30 atoms in length with a

backbone consisting of carbon atoms and optionally one or more heteroatom(s) selected from O, N and S; a linker of 1 to 20 methylene (-CH2-) units; a linker of between 3 and 30 atoms consisting of methylene (-CH2-), ethylene (-C2H4-) and/or propylene (-C3H6-) units and oxygen, wherein the number of ether bonds with oxygen is from 1 to 8, a linker of a backbone of 5 to 30 atoms comprising methylene groups and 1 to 4 carbonyl units bound via ester or amide bond, or a linker of between 11 to 30 atoms comprising 6 to 25 methylene groups, 2 carbonyl units bound via ester or amide bond and in addition 1 to 6 oxygen atoms linked by ether bond.

In one preferred embodiment the linker X in the bis-maleic anhydride according to Formula I for use in a method according to the present invention is selected from the group of linkers consisting of a linker of 1 to 30 atoms in length with a backbone consisting of carbon atoms and optionally one or more heteroatom(s) selected from O, N and S; a linker of 1 to 6 methylene (-CH2-) units; a linker of between 3 and 30 atoms consisting of methylene (-CH2-), ethylene (-C2H4-) and/or propylene (-C3H6-) units and oxygen, wherein the number of ether bonds with oxygen is from 1 to 8, a linker of a backbone of 5 to 30 atoms comprising methylene groups and 1 to 4 carbonyl units bound via ester or amide bond, or a linker of between 11 to 30 atoms comprising 6 to 25 methylene groups, 2 carbonyl units bound via ester or amide bond and in addition 1 to 6 oxygen atoms linked by ether bond.

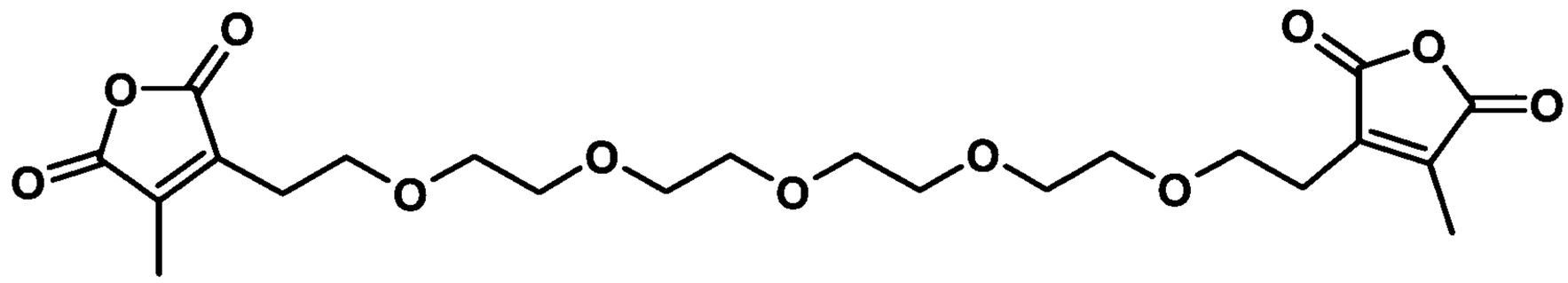
In one preferred embodiment the bis-maleic anhydride according to Formula I for use in a method according to the present invention will have a linker X of 1 to 20 methylene (-CH2-) units. Preferably such type of linker will have 1 to 8 methylene units and also preferred 2 to 6 methylene units.

In a preferred embodiment the present invention relates to a bis-maleic anhydride according to formula I, wherein the linker X consists of five methylene units.

In one preferred embodiment the bis-maleic anhydride according to Formula I for use in a method according to the present invention will have a linker X of between 3 and 30 atoms consisting of methylene (-CH2-), ethylene (-C2H4-) and/or propylene (-C3H6-) units and oxygen, wherein the number of ether bonds with oxygen is from 1 to 6.

An example of such linker is given in Formula II below.

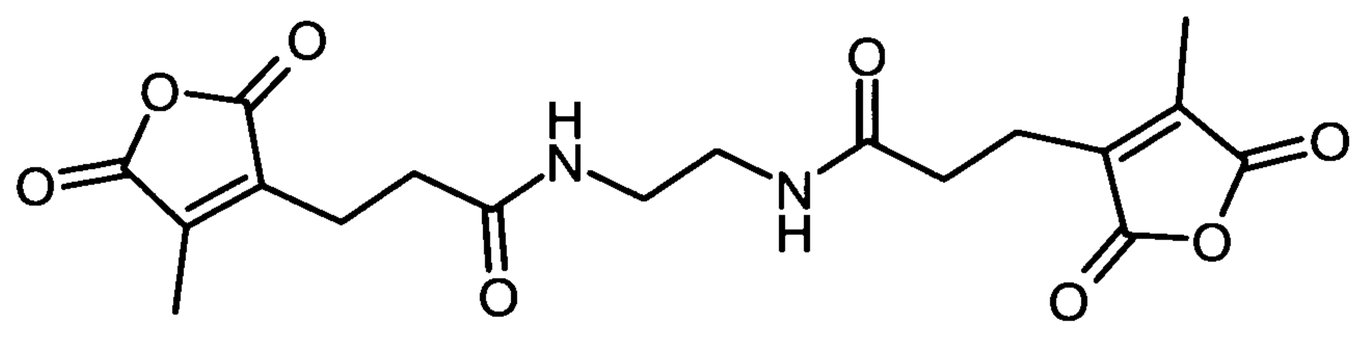
Formula II



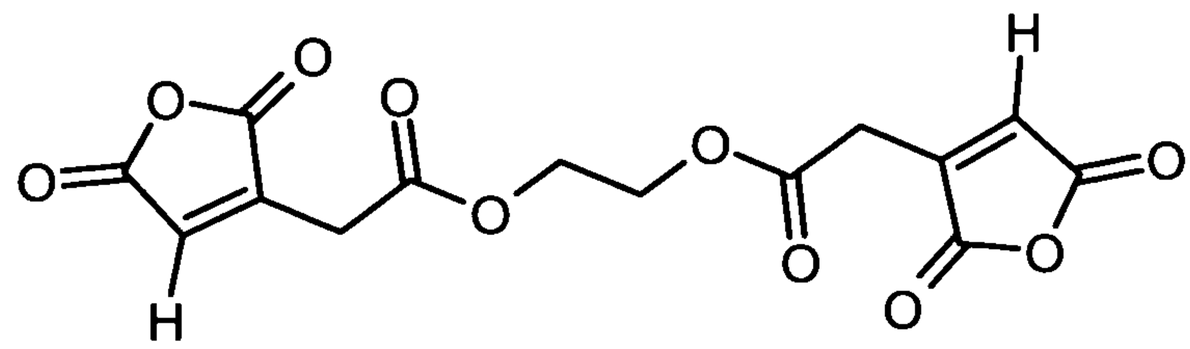
Preferably such linker will comprise from 4 to 8 methylene, ethylene (-C2H4-) and/or propylene (-C3H6-)units and from 1 to 8 ether bonds. Also preferred the linker will have 4 to 6 methylene units and 1 or 2 ether bonds.

In one preferred embodiment the bis-maleic anhydride according to Formula I for use in a method according to the present invention will have a linker X with a backbone of 5 to 30 atoms comprising methylene groups and 1 to 4 carbonyl units bound via ester or amide bond. Also preferred the linker X will have 8 to 20 atoms in length, comprising 4 to 16 methylene groups and 2 to 4 carbonyl units bound via ester or amide bond into the backbone of the linker X. Also preferred the linker X will comprising 4 to 12 methylene groups and 2 to 4 carbonyl units bound via ester or amide bond into the backbone of the linker. In another preferred embodiment the linker X will comprise 4 to 8 methylene groups and 2 carbonyl units bound via ester or amide bond into the backbone of the linker. Examples of such bis-maleic anhydrides are given in Formulas III and IV below.

Formula III

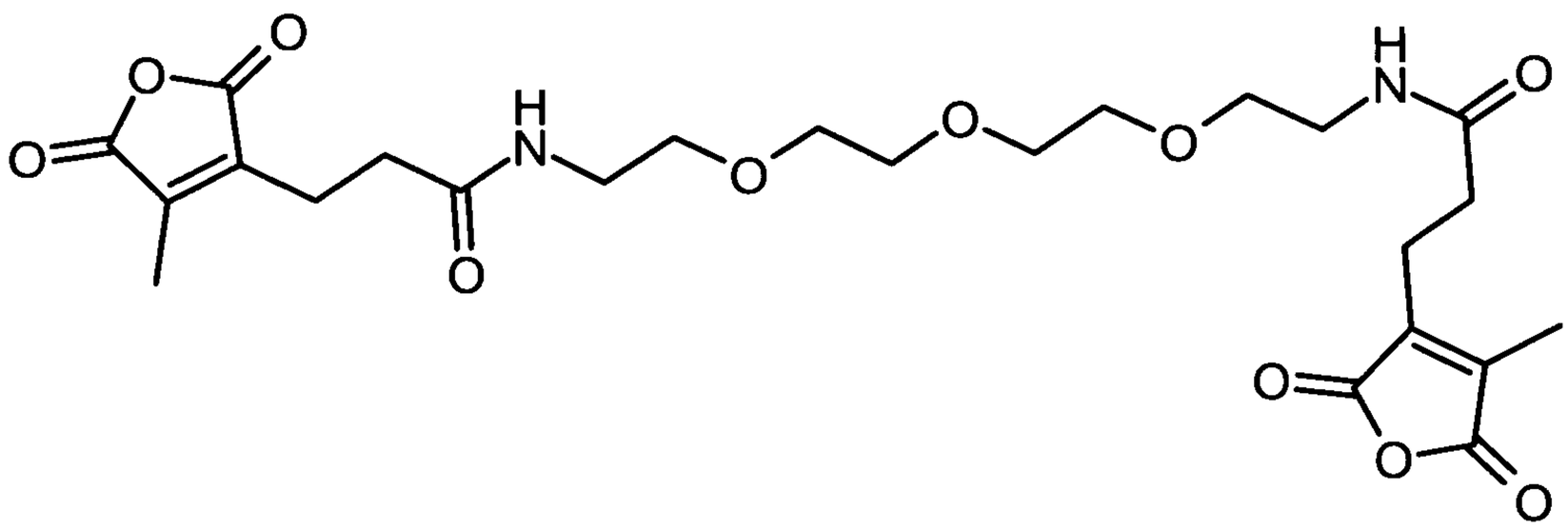


Formula IV

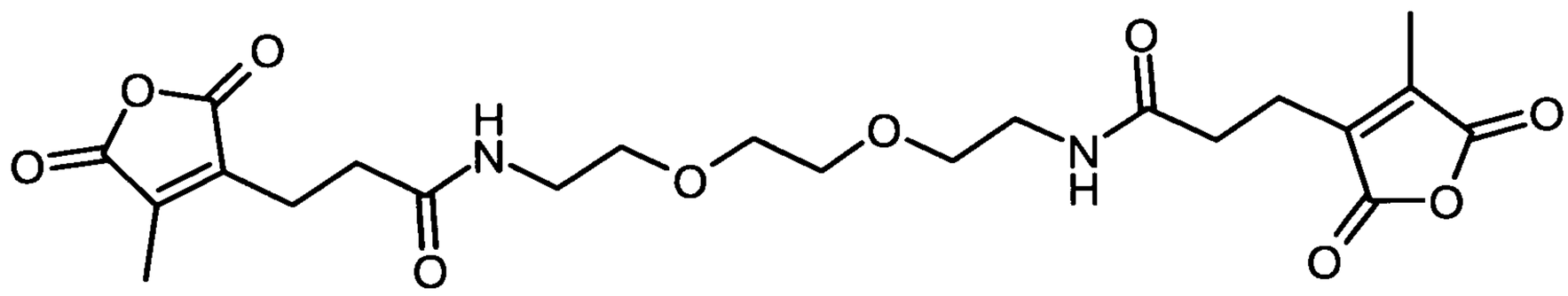


In one preferred embodiment the bis-maleic anhydride according to Formula I for use in a method according to the present invention will have a linker X of between 11 to 30 atoms comprising 6 to 25 methylene groups, 2 carbonyl units bound via ester or amide bond into the backbone of the linker X and in addition 1 to 6 oxygen atoms linked by ether bond. Preferred examples are depicted in Formulas V to VII below.

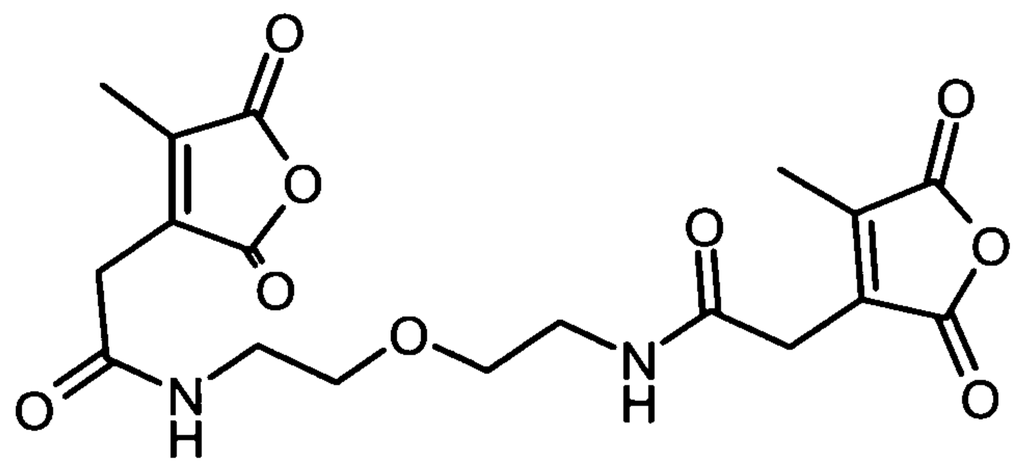
Formula V



Formula VI



Formula VII



The cell or tissue sample may comprise cells derived from an in vitro cell or tissue culture or may represent a sample as available in clinical routine. In a preferred

embodiment the cell or tissue sample will contain cells of interest as investigated in clinical routine. In the field of oncology such cell or tissue sample may e.g. comprise circulating tumor cells or be a tissue suspected or known to contain tumor cells. Preferred samples are whole blood and tissue samples, like specimen obtained by surgery or biopsy.

As the skilled artisan will appreciate, any method according to the present invention is practiced in vitro. The patient sample is stored or discarded after the analysis. The sample is not transferred back into the patient's body.

Cells as e.g. contained in blood are usually at least partially isolated from blood plasma and may be fixed either in suspension or embedded into agar. A sample of tissue, as e.g. obtained by resection or biopsy, usually is either briefly washed in a physiological buffer or directly transferred into a solution containing an appropriate fixative.

As mentioned further above, formaldehyde fixation is a two-step process and therefore not easy to control. The bis-maleic anhydrides as used in a method according to the present invention have the tremendous advantage that they require only one type of reaction to occur, the formation of an amide bond. Once at least two amide bonds are formed between at least two primary amines and maleic anhydride groups, linked to each other via the linker X, thereby cross-linking has occurred. No formation of a methylene bridge in a second type of chemical reaction is required.

Fixation of a cell or tissue sample – despite being probably the most critical step – is in most cases only one out of several potentially critical steps in clinical routine.

Usually the cell or tissue sample is microscopically investigated. To that end a sample has to be prepared that has the appropriate thickness for staining and microscopic investigation. In case of a tissue sample usually a frozen sample or a so-called paraffin block (see below) is cut with a microtome in so-called thin sections. Thin sections usually are 2 to 10 µm thick. In case the sample is a tissue sample the analytic method used in the investigation of such sample preferably is performed on a thin section of the tissue.

In clinical pathology it is routine to take measures that allow for long term storage of a cell or tissue sample. While e.g. tissue preservation can also be obtained by

low temperature storing (e.g. at about -70°C), routine storage conditions are either storage at 4-8°C or even storage at ambient temperature.

After fixation of a cell or tissue sample, in a method as disclosed herein above, direct use of such sample for removal of the bis-maleic anhydride cross-linking agent and for further analysis is possible and represents a preferred embodiment. The sample may be analyzed using any of the methods given below in more detail for FFPE material.

While after fixation of a cell or tissue sample, in a method as disclosed herein above, embedding the fixed sample in paraffin is only one out of several options it is the procedure used most broadly in clinical routine. Paraffin-embedding represents an intermediate step between fixation and analysis.

For long term storage at e.g. ambient temperature it is standard practice that a cell or a tissue sample is dehydrated and embedded in an appropriate medium. The skilled artisan is fully aware of procedural details and these need not to be given here. It is also preferred to practice the methods disclosed in the present application with machines for automatic tissue processing, like embedding, deparaffinizing and/or staining.

In clinical routine paraffin is most widely used to embed and preserve a sample for e.g. later histopathology, immunohistochemistry and so on. The method according to the present invention is compatible with routine methods for embedding in paraffin. Therefore in a preferred embodiment the present invention relates to a method of preserving a cell or a tissue sample the method comprising the steps of

a) fixing a tissue sample with a bis-maleic anhydride cross-linking agent and
b) embedding said fixed sample of step (a) in paraffin.

By this process a paraffin block is obtained that can easily be cut into thin sections. Once embedded, the cell or tissue sample may be stored till an analysis fall due. The analysis can be performed within hours or days or as the case may be after several years. Before a later analysis can be performed, on e.g. a section of paraffin embedded tissue, it is necessary to remove the paraffin and to rehydrate the sample of interest. Various methods for removal of paraffin are available and the skilled artisan will have no difficulty to remove paraffin in an appropriate method.

As mentioned above, various types of analyses may be performed on a cell or a tissue sample. Usually morphology, enzymatic activity, immunoreactivity and/or nucleic acids is/are assessed. As will be appreciated each of these types of assessments will to a large extend depend on the degree of structural and functional preservation of the sample to be investigated.

Investigations on enzymatic properties usually are not at stake if formaldehyde is used as a fixative, because of the often observed negative effects of formaldehyde on enzymatic activity. Due to the gentle fixation in a method as disclosed in the present invention it is more likely that enzymatic activity is less or as the case may be even not affected. In a preferred embodiment the present invention relates to a method for analyzing enzymatic activity in a cell or tissue sample the method comprising the steps of fixing the sample with a bis-maleic anhydride cross-linking agent, removing the bis-maleic amide cross-link and analyzing the enzymatic activity.

The method of fixation with a bis-maleic cross-linking agent according to the present application can be used with great advantage in routine procedures of immunohistochemistry. In a preferred embodiment the present invention thus relates to a method for performing immunohistochemistry on a cell or a tissue sample the method comprising the steps of

a) fixing a cell or tissue sample with a bis-maleic anhydride cross-linking agent,
b) embedding said fixed sample of step (a) in paraffin
c) de-paraffinizing said sample,
d) removing the bis-maleic amide cross-link and
e) immunologically detecting an epitope of interest.

In one embodiment the invention relates to a method comprising fixing a cell or tissue sample as disclosed in the present invention the method further comprising the steps of

a) embedding the fixed sample in paraffin
b) de-paraffinizing said sample,
c) removing the bis-maleic amide cross-link and
e) immunologically detecting an epitope of interest.

If a cell or tissue sample is fixed with a bis-maleic cross-linking agent and the bis-maleic amide cross-link is released before analysis, the primary amine as originally present in the sample becomes available again. It is expected that this will represent a major advantage over other fixatives, like formaldehyde, that are known for their often detrimental and irreversible effects on many epitopes. This negative may be especially critical if the single epitope recognized by a monoclonal antibody is affected or destroyed. Many of these potentially extremely valuable monoclonal antibodies have gained little attention and market penetration because they do not work in standard immunohistochemistry based on formalin-fixed paraffin embedded tissue (FFPET). It is quite likely that many monoclonal antibodies failing with FFPET will work on a sample fixed with a bis-maleic anhydride cross-linking agent. In a preferred embodiment the present invention therefore relates to an immunohistochemistry method essentially as described in the previous paragraph with the additional feature that the epitope of interest is an epitope that is masked or destroyed when said cell or tissue sample comprising said epitope is fixed with a formaldehyde fixative. In other words, the method is practiced using an antibody not working with FFPET.

As decribed above the method disclosed in the present application works well with polypeptides having e.g. enzymatic or antigenic properties. Surprisingly the method disclosed herein also is of advantage in the detection of a nucleic acid of interest.

In one preferred embodiment the nucleic acid is a deoxyribonucleic acid (DNA) as for example present in the nucleus of a eukaryotic cell. DNA in one preferred embodiment is analyzed by an in situ hybridization method. Methods for in situ hybridization (ISH) are well-known to the skilled artisan. Gene amplification can e.g. be measured with in situ hybridization methods, like fluorescence in situ hybridization techniques (FISH), chromogenic in situ hybridization techniques (CISH) or silver in situ hybridization techniques (SISH). In a preferred embodiment the present invention relates to a method for detecting in vitro a nucleic acid of interest by in situ hybridization on a cell or a tissue sample the method comprising the steps of

a) fixing a cell or tissue sample with a bis-maleic anhydride cross-linking agent,
b) embedding said fixed sample of step (a) in paraffin,
c) de-paraffinizing said sample,
d) removing the bis-maleic amide cross-link and

e) detecting a nucleic acid of interest by in situ hybridization.

In one embodiment the invention relates to a method comprising fixing a cell or tissue sample as disclosed in the present invention the method further comprising the steps of

a) embedding the fixed sample of step (a) in paraffin
b) de-paraffinizing said sample,
c) removing the bis-maleic amide cross-link and
d) detecting a nucleic acid of interest by in situ hybridization.

Surprisingly the fixation method as described in the present invention also is of advantage in the detection of m-RNA in a cell or tissue sample prepared used a bis-maleic anhydride cross-linking agent. The expression level of an m-RNA of interest may be determined by appropriate techniques, such as Northern Blot, real time polymerase chain reaction (RT-PCR) and the like. All these detection techniques are well known in the art and can be deduced from standard text books, such as Lottspeich (Bioanalytik, Spektrum Akademischer Verlag, 1998) or Sambrook and Russell (2001, Molecular Cloning: A Laboratory Manual, CSH Press, Cold Spring Harbor, NY, USA). Preferably m-RNA is detected using real time polymerase chain reaction (RT-PCR). In a preferred embodiment the present invention therefore relates to a method for detecting in vitro a nucleic acid of interest by RT-PCR in a cell or a tissue sample the method comprising the steps of

a) fixing a cell or tissue sample with a bis-maleic anhydride cross-linking agent by a method as described herein above,
b) embedding said fixed sample of step (a) in paraffin
c) de-paraffinizing said sample,
d) removing the bis-maleic amide cross-link and
e) detecting a nucleic acid of interest by performing RT-PCR.

In one embodiment the invention relates to a method comprising fixing a cell or tissue sample as disclosed in the present invention the method further comprising the steps of

a) embedding the fixed sample in paraffin
b) de-paraffinizing said sample,
c) removing the bis-maleic amide cross-link and

d) detecting a nucleic acid of interest by performing RT-PCR.

In a further preferred embodiment a nucleic acid is isolated from a cell or tissue sample that had been fixed using a bis-maleic anhydride cross-linking agent and the isolated nucleic acid is further analyzed. Further preferred such isolated nucleic acid is used for mutation analysis. In a preferred embodiment the present invention therefore relates to a method for performing a mutation analysis in vitro on an nucleic acid sample isolated from a cell or a tissue sample the method comprising the steps of

a) fixing a cell or tissue sample with a bis-maleic anhydride cross-linking agent,
b) embedding said fixed sample of step (a) in paraffin,
c) de-paraffinizing said sample,
d) removing the bis-maleic amide cross-link,
e) isolating the nucleic acid and
f) performing mutation analysis using the nucleic acid isolated in step (e).

In one embodiment the invention relates to a method comprising fixing a cell or tissue sample as disclosed in the present invention the method further comprising the steps of

a) embedding the fixed sample in paraffin,
b) de-paraffinizing said sample,
c) removing the bis-maleic amide cross-link,
d) isolating the nucleic acid and
e) performing mutation analysis using the nucleic acid isolated in step (d).

Determining the presence or absence of a particular mutation can be performed in a variety of ways. Such methods including but not limited are PCR, hybridization with allele-specific probes, enzymatic mutation detection, chemical cleavage of mismatches, mass spectrometry or DNA sequencing, including minisequencing. In particular embodiments, hybridization with allele specific probes can be conducted in two formats: (1) allele specific oligonucleotides bound to a solid phase (glass, silicon, nylon membranes) and the labeled sample in solution, as in many DNA chip applications, or (2) bound sample (often cloned DNA or PCR amplified DNA) and labeled oligonucleotides in solution (either allele specific or short so as to allow sequencing by hybridization). Preferably, the determination of the presence

or absence of a mutation involves determining an appropriate nucleotide sequence comprising the site of mutation by methods such as polymerase chain reaction (PCR), DNA sequencing, oligonucleotide hybridization or mass spectrometry.

With the method now at hand by the disclosure provided in the present application it is even possible to detect a nucleic acid of interest and a polypeptide of interest in the same sample. In a further preferred embodiment the present invention relates to a method for detecting in vitro at least one polypeptide of interest by immunohistochemistry and at least one nucleic acid of interest in one test sample comprising a cell or a tissue sample, the method comprising the steps of

a) fixing a cell or tissue sample with a bis-maleic anhydride cross-linking agent,
b) embedding said fixed sample of step (a) in paraffin,
c) de-paraffinizing said sample,
d) removing the bis-maleic amide cross-link and
e) immunologically detecting the at least one polypeptide of interest and detecting the at least one nucleic acid of interest by performing RT-PCR or fluorescence in situ hybridization.

In one embodiment the invention relates to a method comprising fixing a cell or tissue sample as disclosed in the present invention the method further comprising the steps of

a) embedding the fixed sample in paraffin,
b) de-paraffinizing said sample,
c) removing the bis-maleic amide cross-link and
d) immunologically detecting the at least one polypeptide of interest and detecting the at least one nucleic acid of interest by performing RT-PCR or fluorescence in situ hybridization.

As discussed above and as further illustrated in the Examples given, the use of a bis-maleic anhydride cross-linking agent has great advantages in many respect over other fixatives and procedures used in the routine of an up-to-date pathology laboratory. In a very preferred embodiment the present invention thus relates to the use of a bis-maleic anhydride cross-linking agent for fixation of a cell or a tissue sample.

In yet a further preferred embodiment a bis-maleic anhydride cross-linking agent is used in the preparation of a ready-to-use fixative. Preferably the present invention thus relates to the use of a bis-maleic anhydride in the manufacturing of a fixative for fixation of a cell or tissue sample.

Preferably the bis maleic anhydride cross-linking agent used for fixation of a cell or a tissue sample or in the manufacturing of a fixative for fixation of a cell or tissue sample will be a bis-maleic cross-linking agent as defined in Formula I. Preferably the linker X of Formula I will be selected from the group of linkers disclosed as preferred when practicing the fixation method disclosed in the present invention. In a further embodiment a cross-linking agent will be selected from a compound as described in Formula II, III, IV, V, VI, VII, VIII and IX.

Now that the tremendous advantages of using a bis-maleic anhydride cross-linking agent, especially its easy to accomplish reversibility are known, it can easily be imagined that such reagent can be combined with other fixatives, e.g. with a fixative bringing about a permanent fixation. This way it may be possible to further improve the long term preservation of a tissue, but yet to have the possibility to regain at least a relevant portion of a nucleic acid or an antigen of interest easily.

In another preferred embodiment the present application relates to a method of fixing a cell or a tissue sample wherein a mixture comprising a bis-maleic acid anhydride cross-linking agent and a second cross-linking agent selected from formaldehyde and/or glutardialdehyde is used. Preferably such mixture is one as described hereinafter. In yet another preferred embodiment the present application relates to a fixative comprising a bis-maleic acid anhydride cross-linking agent and a fixative selected from formaldehyde and/or glutardialdehyde. The other components of such fixative will be selected from the preferred embodiments given for a bis-maleic anhydride fixative above. Preferably the volume-based ratio of the bis-maleic anhydride cross-linking agent to either formaldehyde or glutardialdehyde or to the sum of both if a mixture is used will be from 1:10 to 10:1 (weight/weight). The final concentration of the sum of fixatives in such mixture will be as outlined above for a fixative containing only a bis-maleic anhydride cross-linking agent. Preferably such fixative comprising a bis-maleic anhydride cross-linking agent and either formaldehyde or glutardialdehyde or both will have a pH in the range of from pH 8.0 to 11.0.

The following examples, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figure 1 shows stained 4 µm thin sections obtained from H322M xenograft tumor bearing SCID beige mice stained with monoclonal antibody 5G11 that specifically binds to IGF-1R. Tissue fixation had been performed with different fixatives using 4% formaldehyde ("formalin"), 4% or 8% bis-citraconic acid, respectively. Immunohistochemical localization of IGF-1R after heat-induced antigen retrieval for formaldehyde-fixed tissue and removal of bis-citraconic acid from tissue fixed with bis-citraconic acid by use of an acidic buffer, respectively, demonstrated comparable staining intensity or quality between the fixatives formalin (Fig. 1; A) or bis-citraconic acid used at 4% (Fig. 1: B) and 8% (Fig. 1: C), respectively.

Figure 2 shows stained 4 µm thin sections obtained from H322M xenograft tumor bearing SCID beige mice stained with monoclonal antibody 3C6 that specifically binds to EGFR. Tissue fixation had been performed with different fixatives using 4% formaldehyde ("formalin"), 4% or 8% bis-citraconic acid, respectively. Immunohistochemical localization of EGFR after protease-assisted antigen retrieval for formaldehyde-fixed tissue and removal of bis-citraconic acid from tissue fixed with bis-citraconic acid by use of an acidic buffer, respectively, , demonstrated comparable staining intensity or quality between the fixatives formalin (Fig. 2; A) or bis-citraconic acid used at 4% (Fig. 2: B) and 8% (Fig. 2: C), respectively.

Figure 3 shows the PCR-amplification of the EGFR-gene using mRNA isolated from MDA468 cells that had been subjected to different pre-treatment/fixation protocols. Shown are EGFR-mRNA amplifications from MDA-MB468 cells (fresh and stored for 4 hours in RPMI both not fixed, fresh MDA-MB468 cells treated/fixed in 10% DMSO, 80% DMSO, 10% DMSO with 1%

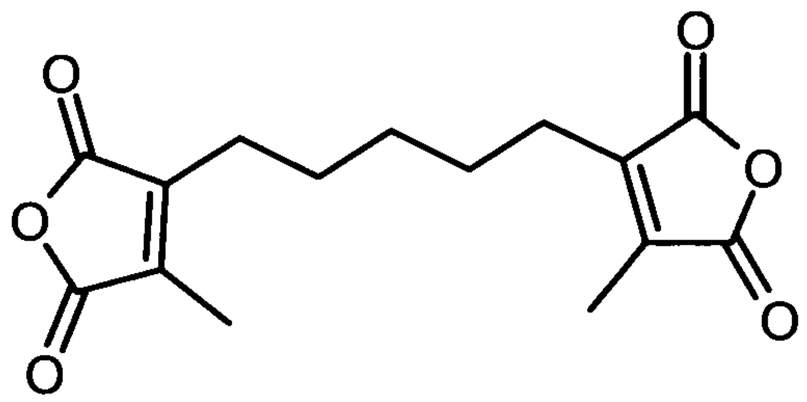
bis-citraconic acid, 80% DMSO with 1% bis-citraconic acid and water "Wasser" as negative control, respectively). As obvious from the amplification curves and the inserted table, mRNA from all samples is amplified in a rather similar manner.

Figure 4 shows the PCR-amplification of the HER2-gene using mRNA isolated from MDA468 cells that had been subjected to different pre-treatment/fixation protocols. Shown are HER2-mRNA amplifications from MDA-MB468 cells (fresh and stored for 4 hours in RPM both not fixed, fresh MDA-MB468 cells treated/fixed in 10% DMSO, 80% DMSO, 10% DMSO with 1% bis-citraconic acid, 80% DMSO with 1% bis-citraconic acid and water "Wasser" as negative control, respectively. As obvious from the amplification curves and the inserted table, mRNA from all samples is amplified in a rather similar manner.

Example 1:

Synthesis of "bis-citraconic acid" of Formula VIII

Formula VIII



Synthesis of methyl-3-tolylcarbamoyl-acrylic acid

To a solution of 3,2 ml 3-methyl-furan-2,5-dione (citraconic anhydride) in 25 ml ethyl ether a solution of 3,74 g p-tolylamine in 25 ml ethyl ether was added dropwise over a period of 15 min.. The yellow suspension was stirred for 1 h and filtrated. The residue was washed with ethyl ether and dried under vacuum.

Yield: 7,22 g, 94%

Synthesis of 3-methyl-1-p-tolyl-pyrrole-2,5-dione

7,22 g of methyl-3-tolylcarbamoyl-acrylic acid were suspended in 60 ml acetic anhydride. The suspension was heated under reflux for 3 h . After cooling to room

temperature the solvent was removed under vacuum. The residue was recrystallized from ethanol.

Yield: 4,32 g, 65 %

Synthesis of 3-(5-(4-methyl-2,5-dione-1-p-tolyl-2,5-dihydro-1H-pyrrol-3-yl)-pentyl-)-4-methyl-1-p-tolyl-pyrrole-2,5-dione

12 g 3-methyl-1-p-tolyl-pyrrole-2,5-dione and 15,6 g triphenyl-phosphane are dissolved in 155 ml acetic acid and 2,15 ml pentanedial are added. The reaction mixture was refluxed for 20 h. The acetic acid was removed by distillation and the residue heated up to 150-160°C for 6 h.

The crude product was purified by column chromatography on silica gel, petrolether:acetic acid ethyl ester 7:3. The product was further purified by digestion in methanol, filtrated and dried.

Yield: 2 g, 35%

Synthesis of 3-(5-(4-methyl-2,5-dioxo-2,5-dihydro-furan-3-yl)-pentyl)-4-methyl-furan-2,5-dione ("bis-citraconic acid")

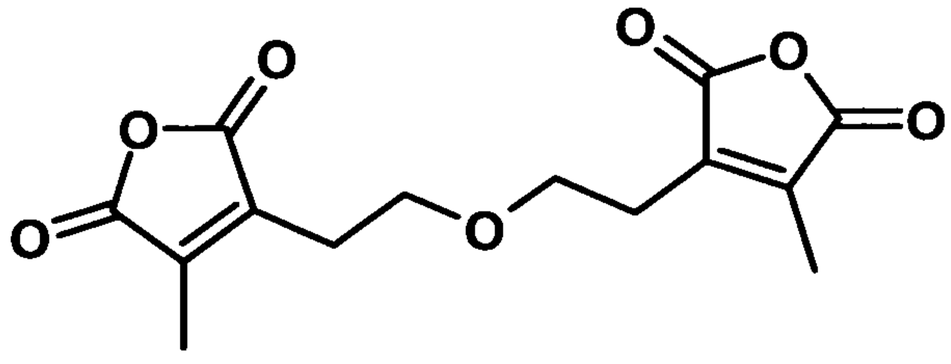
1,07 g of 3-(5-(4-methyl-2,5-dione-1-p-tolyl-2,5-dihydro-1H-pyrrol-3-yl)-pentyl-)-4-methyl-1-p-tolyl-pyrrole-2,5-dione were dissolved in 30 ml of a 1:1 mixture of tetrahydrofuran and methanol. After the addition of 3,48 g potassium hydroxide dissolved in water the mixture was heated under reflux for 3 h. The solvent was removed by distillation and the residue was purified by column chromatography on silica gel, petrolether:acedic acid ethyl ester 7:3. The product was dried under vacuum.

Yield: 402 mg, 60%

Example 2:

Synthesis of 3-(5-(4-methyl-2,5-dioxo-2,5-dihydro-furan-3-yl)-3-oxa-pentyl)-4-methyl-furan-2,5-dione (Formula IX):

Formula IX



The cross-linking agent 3-(5-(4-methyl-2,5-dioxo-2,5-dihydro-furan-3-yl)-3-oxa-pentyl)-4-methyl-furan-2,5-dione (Formula IX) is synthesized analogous to the procedure described in Example 1 using 3-oxa-1,5-pentanedial instead of 1,5-pentanedial. The starting material 3-oxa-1,5-pentanedial is described by Bowers, S. et al., Bioorganic & Medicinal Chemistry Letters 19 (2009) 6952-6956.

Example 3:

Staining with an antibody to IGF-1R

H322M xenograft tumor bearing SCID beige mice were sacrificed. The tumors were removed and were cut into 3 pieces of approximately the same size. The tissue samples were subsequently transferred into the respective fixation solutions. For fixation with bis-citraconic acid this substance was resolved in DMSO to a final concentration of 80 % by repeated pipetting at room temperature. After complete dissolution this solution was either used directly or diluted 1:1 in DMSO and further diluted in 1 × PBS pH 7,4, resulting in PBS with a final concentration of 10% DMSO and 8 % or 4 % of bis-citraconic acid, respectively. To test the impact of different concentrations of bis-citraconic acid on fixation efficacy, solutions containing 4% or 8% bis-citraconic acid (w/v) were prepared. For preparation of the formaldehyde fixative, formalin (40% (w/v) paraformaldehyde in H_2O) was diluted 1:10 in 1 × PBS pH 7.4.

All tumor samples were fixed over night for 12 h at room temperature. The next day the tissue samples were washed with H_2O for 1 h. Afterwards the fixed tumor tissues were embedded in paraffin.

Sections of the paraffin embedded tissue samples fixed with different fixatives (4% formalin, 4% or 8% bis-citraconic acid) were cut at 4 µm using a conventional

rotation microtome. For immunohistochemical localization of IGF-1R the cut tissue sections were mounted on glass slides. Deparaffinization of the tissue samples was performed on the Ventana Benchmark XT automated IHC stainer (Ventana, Tucson). For localization of IGF-1R with the <IGF-1R> 5G11 monoclonal antibody (Ventana, Tucson) in FFPE tissue by immunohistochemistry, a heat induced antigen retrieval has to be performed prior to staining of the formalin fixed sample. Heat induced antigen retrieval for immunohistochemical detection of IGF-1R was performed by incubating the tissue sections on the Ventana Benchmark XT for 1 h at 95°C in buffer CC1 (Ventana, Tucson). Antigen retrieval in thin sections previously fixed with bis-citraconic acid and after paraffin can be performed by a simple incubation of the tissue sections in a buffer with an acidic pH. Thin sections were incubated in buffer of pH 5.8 for 2 h. After antigen retrieval, all slides were placed on the Ventana Benchmark and were stained for IGF-1R with a primary antibody incubation time of 16 min. The bound primary antibody was detected using Ventana *iview* DAB detection kit. Examination of the stained sections revealed that fixation of tissues with bis-citraconic acid conserved the morphology of the tissue (Fig. 1 B and C). Furthermore, the bis-citraconic acid could be retrieved by a simple incubation in an acidic buffer solution. Immunohistochemical localization of IGF-1R did not reveal great differences in staining intensity or morphological quality between formalin (Fig. 1; A) or bis-citraconic acid fixed tissues (Fig. 1; B and C).

The results obtained in this Example demonstrate that fixation with bis-citraconic acid enables conservation as well as easy and gentle retrieval of epitopes that in formalin-fixed tissues only become accessible after tissue treatment with a method known as heat-induced antigen retrieval.

Example 4:

Staining with an antibody to EGFR

By a procedure similar to Example 2, formalin or bis-citraconic acid fixed tissues were prepared for staining for EGFR using the antibody 3C6 (<EGFR> mAB 3C6; Ventana, Tucson). This antibody is known to depend on a protease pretreatment of FFPE-derived tissue sections in order to regain access to its epitope in such FFPE-sample. As shown in Figure 2, no differences in immunohistochemical localization of EGFR between formalin fixation and protease-assisted epitope retrieval or bis-citraconic acid fixation and retrieval by incubation in an acidic buffer were found.

The results obtained in this Example demonstrate that fixation with bis-citraconic acid enables conservation as well as easy and gentle retrieval of epitopes that in formalin-fixed tissues only become accessible after tissue treatment with a protease.

As demonstrated in Examples 2 and 3, collectively, bis-citraconic acid fixation and retrieval is the method for the detection of different epitopes which so far in formalin-fixed tissue have to be retrieved by one or more different retrieval methods (heat or protease induced). Bis-citraconic acid, as a prototype for other bis-maleic anhydrides, works without harsh retrieval methods for different antibodies otherwise requiring quite different methods of retrieval. Whereas heat-induced or protease-assisted retrieval is not easy to standardize and reproduce, it will be possible to obtain a more reproducible accessibility/reactivity of antigens/epitopes by use of a gentle and easy to remove fixative based on a bis-maleic anhydride cross-linking agent as shown above.

Example 5:

DNA isolation and qPCR for gene amplification analysis

MDA-MB468 cells were first fixed in different fixation reagents for 10 min and then neutralized in citrate buffer (pH 4.4). The different samples given in Figure 3 are MDA-MB468 cells (fresh and stored for 4 hours in RPM both not fixed, fresh MDA-MB468 cells treated/fixed in 10% DMSO, 80% DMSO, 10% DMSO with 1% bis-citraconic acid and 80% DMSO with 1% bis-citraconic acid, respectively.

After the different fixation procedures DNA isolation was performed using 1 x 10^7 MDA-MB468 cells. To isolate the DNA the High Pure Template Preparation Kit (Roche Diagnostics GmbH, Cat. No.: 11796828) was used according to the manufacturer´s instructions. Isolated DNA was stored at -20°C.

Amplification status of the EGFR and HER2 was measured in MDA-MB468 cells. Therefore a quantitative PCR based on the use of hydrolysis probes (Taqman probes) was performed using gene specific primers and probes (see Table 1). The probes for the target genes were labeled with Fam at the 5`end and with BHQ-2 at the 3`end.

Table 1: Primer and Probes for the target genes HER2 and EGFR

	HER2	EGFR
Forward Primer:	CTCAGCGTACCCTTGTCC SEQ ID NO:1	TGAAAACACCGCAGCATGTCAA SEQ ID NO:4
Reverse Primer:	TGTCAGGCAGATGCCCAGA SEQ ID NO:2	CTCCTTCTGCATGGTATTCTTTCTCT SEQ ID NO:5
Probes:	TGGTGTGGGCTCCCCATATGTCTCCC SEQ ID NO:3	TTTGGGCTGGCCAAACTGCTGGGTG SEQ ID NO:6

For each gene an individual, verified PCR- mix was used (see Table 2).

Table 2: PCR-Mix composition for the qPCR assays

PCR-Mix for EGFR, HER2:	
component	1x (µl)
Nuclease-Free H2O	**5.5**
5x RNA MasterMix	**4.0**
Forward Primer (500 nM)	**1.0**
Reverse Primer (500 nM)	**1.0**
TaqMan Probe (100 nM)	**0.2**
DMSO 100%	**0.8**
MgAc (25 mM)	**2.2**
template	**5.0**

Each mix is composed of 5 µM forward and reverse primer and 2 µM probe. The applied Z05 polymerase was included in the COBAS Taqman RNA Reaction Mix (LUO M/N 58004938) purchased by Roche Molecular Diagnostics (Branchburg, USA) and Magnesium Acetate [25 mM] (Fluka, Cat. No.: 63049) was added in different concentrations according to the different oligo mixes. 2 µl DNA-template was used and filled up to 5 µl with nuclease free water. Each sample was measured in triplicates. The LightCycler 480 (Roche Diagnostics GmbH) and appropriate 96-well plates and sealing foils were applied for the measurements. The following thermocycling profile was used on the cycler (Table 3).

Table 3: LightCycler thermocycling profile

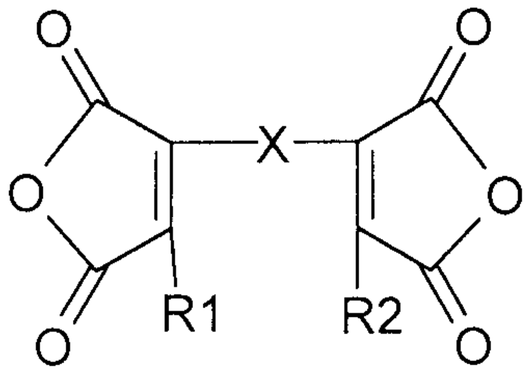
Program Name	Cycles	Analysis Mode
Decontamination	1	Quantification
Amplification	47	Quantification
Cooling	1	None

Target [°C]	Acquisition Mode	Hold	Ramp Rate [°C/s]
50	None	5 min	4,4
95	None	1 min	4,4
Amplification			
92	None	15s	4,4
60	Single	50s	2,2
Cooling			
40	None	30s	2,2

Patent Claims

1. A method for fixation of a cell or a tissue sample in vitro wherein said cell or said tissue sample is incubated with a bis-maleic anhydride according to Formula I,

Formula I



wherein R1 and R2 independently are selected from the group consisting of hydrogen, methyl, ethyl, propyl, isopropyl and butyl,

wherein X is a linker with between 1 and 30 atoms in length,

whereby said cell or said tissue sample is fixed.

2. The method of claim 1, wherein in the bis-maleic anhydride R1 is hydrogen or methyl and R2 is hydrogen or methyl.

3. The method of claim 1 or 2, wherein in the bis-maleic anhydride R1 and R2 are the same.

4. The method of claim 1, 2 or 3, wherein in the bis-maleic anhydride the linker X is 1 to 20 atoms in length.

5. The bis-maleic anhydride according to any one of claims 1 to 4, wherein the backbone of the linker X consists of carbon atoms and optionally one or more heteroatom(s) selected from O, N and S.

6. The method according to any one of claims 1 to 5, wherein the sample is incubated with the bis-maleic anhydride for 1 to 72 hours.

7. The method according to any one of claims 1 to 6 the method further comprising the step of embedding the fixed sample in paraffin.

8. The method according to any one of claims 1 to 6, the method further comprising the steps of

a) embedding the fixed sample in paraffin
b) de-paraffinizing said sample,
c) removing the bis-maleic amide cross-link and
e) immunologically detecting an epitope of interest.

9. The method of claim 8, wherein the epitope of interest is an epitope that is masked or destroyed when said cell or tissue sample comprising said epitope is fixed with a formaldehyde fixative.

10. The method according to any one of claims 1 to 6, the method further comprising the steps of

a) embedding the fixed sample of step (a) in paraffin
b) de-paraffinizing said sample,
c) removing the bis-maleic amide cross-link and
d) detecting a nucleic acid of interest by in situ hybridization.

11. The method according to any one of claims 1 to 6, the method further comprising the steps of

a) embedding the fixed sample in paraffin
b) de-paraffinizing said sample,
c) removing the bis-maleic amide cross-link and
d) detecting a nucleic acid of interest by performing RT-PCR.

12. The method according to any one of claims 1 to 6, the method further comprising the steps of

a) embedding the fixed sample in paraffin,
b) de-paraffinizing said sample,

c) removing the bis-maleic amide cross-link and

d) immunologically detecting a polypeptide of interest and detecting a nucleic acid of interest by performing RT-PCR or fluorescence in situ hybridization.

13. The method according to any one of claims 1 to 6, the method further comprising the steps of

a) embedding the fixed sample in paraffin,

b) de-paraffinizing said sample,

c) removing the bis-maleic amide cross-link,

d) isolating a nucleic acid and

e) performing mutation analysis using the nucleic acid isolated in step (d).

14. Use of a bis-maleic anhydride cross-linking agent according to Formula I, as defined in claim 1, for fixation of a cell or a tissue sample.

15. Use of a bis-maleic anhydride cross-linking agent according to Formula I, as defined in claim 1, in the manufacturing of a fixative for fixation of a cell or tissue sample.

Fig. 1

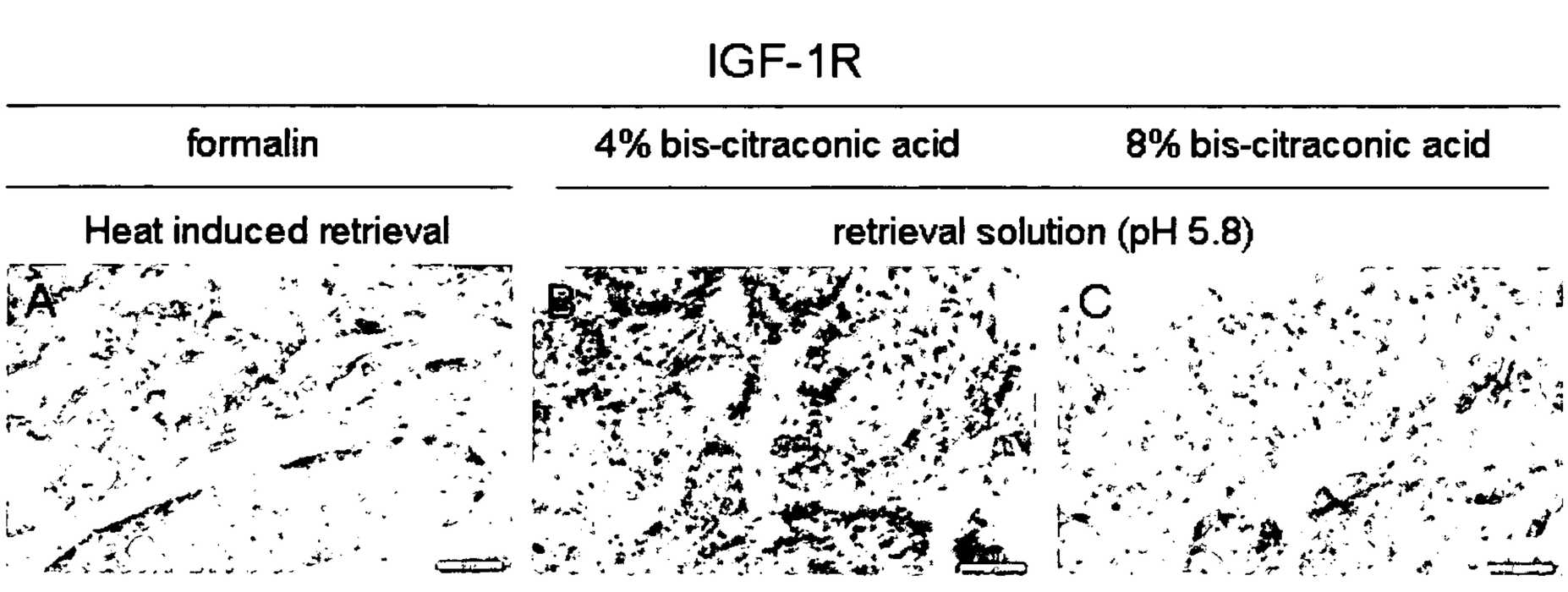

IGF-1R
formalin
4% bis-citraconic acid
8% bis-citraconic acid
Heat induced retrieval
retrieval solution (pH 5.8)
A
B
C

Fig. 2

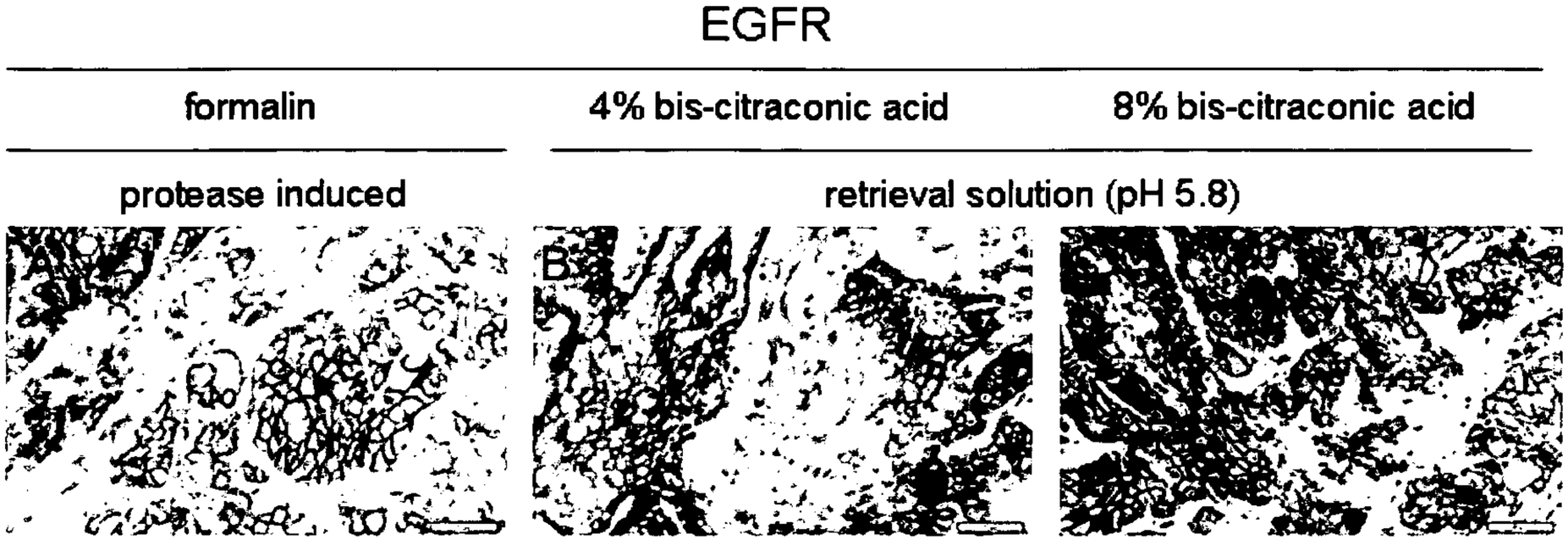

EGFR
formalin
4% bis-citraconic acid
8% bis-citraconic acid
protease induced
retrieval solution (pH 5.8)
A
B

Fig. 3
EGFR

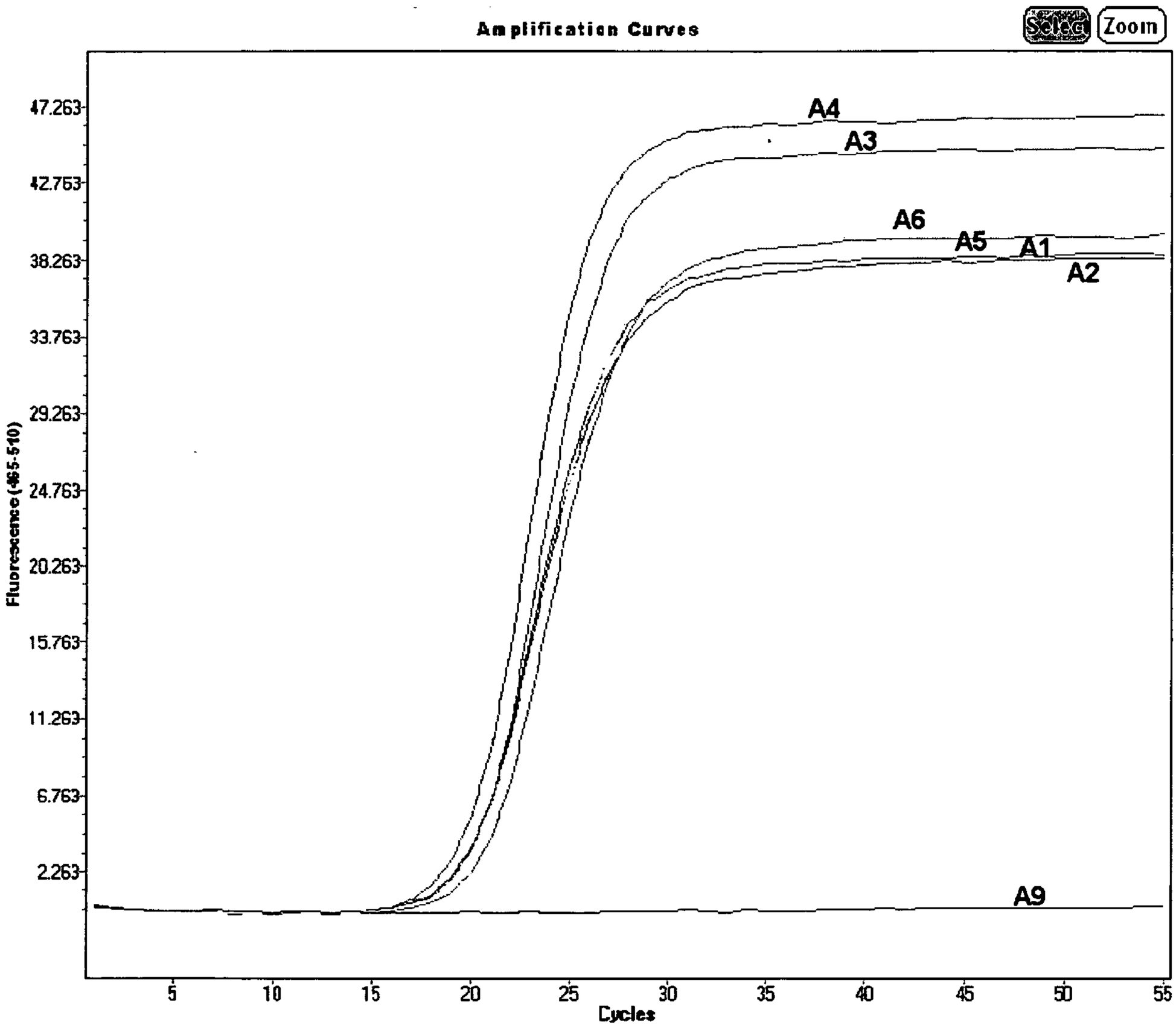
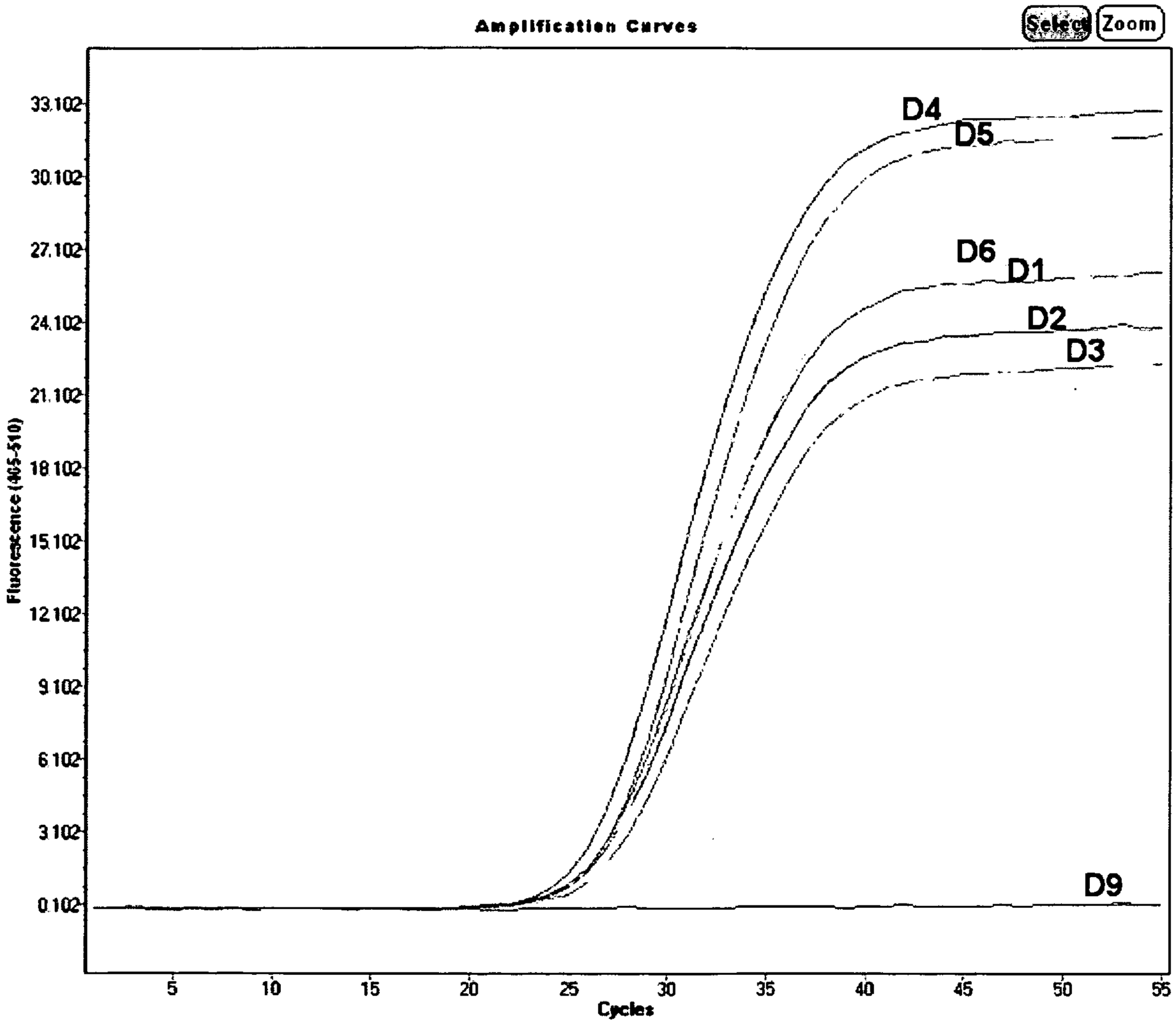

Amplification Curves
Select
Zoom
47.263
42.763
38.263
33.763
29.263
24.763
20.263
15.763
11.263
6.763
2.263
Fluorescence (465-510)
A4
A3
A6
A5
A1
A2
A9
5
10
15
20
25
30
35
40
45
50
55
Cycles
A1 MDA468 frisch 19.90
A2 MDA468 4h in RPMI 19.92
A3 10% DMSO 20.23
A4 80% DMSO 19.64
A5 1% AD264 in 10%DMS 20.69
A6 1% AD264 in 80%DMS 20.24
A9 Wasser

Fig. 4

HER2



■	D1 MDA468 frisch	26.81
■	D2 MDA468 4h in RPMI	26.89
■	D3 10% DMSO	27.32
■	D4 80% DMSO	26.34
■	D5 1% AD264 in 10%DMS	27.08
	D6 1% AD264 in 80%DMS	27.06
■	D9 Wasser	

EGFR

formalin | 4% bis-citraconic acid | 8% bis-citraconic acid

protease induced | retrieval solution (pH 5.8)